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A COMPARATIVE STUDY REGARDING THE ASSOCIATION OF ALPHA-2U GLOBULIN WITH THE NEPHROTOXIC MECHANISM OF CERTAIN PETROLEUM-BASED AIR FORCE FUELS

AFOSR 84-0283

FINAL REPORT

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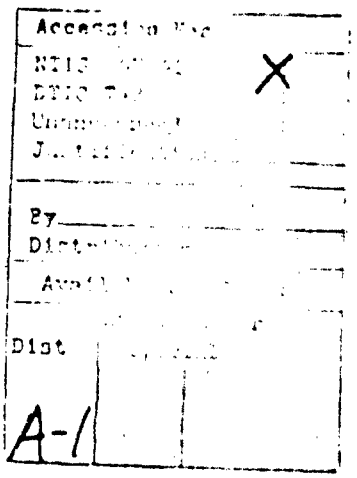
October, 1986



ABSTRACT

Fischer 344 male rats have a dose and time dependent proximal tubular degeneration, induced by certain petroleum-based fuels. This degeneration may be associated with a low molecular weight alpha globulin termed alpha-2U globulin. A new method was developed to obtain monospecific immunologic reagents for alpha-2U globulin using diafiltration, anion-exchange and hydroxylapatite chromatography. Rocket immunoelectrophoretic and isoelectric focusing techniques were developed to quantitatively and qualitatively assess changes in alpha-2U globulin after experimental exposure to hydrocarbon compounds.

INTRODUCTION

Preliminary studies at AAMRL/THT, Wright-Patterson Air Force Base, suggested that a sexually dimorphic urinary protein (alpha-2U globulin) in the adult, male Fischer 344 rat might be involved in a hydrocarbon-induced nephrotoxic response. The principal investigator, in collaboration with toxicologists at AAMRL/THT designed this project to establish scientifically valid methods to evaluate certain potentially hazardous elements of petroleum-based Air Force fuels. The project was designed in two phases.

Phase I centered on the development of monospecific immunologic reagents to investigate the role of A2U in the nephrotoxic event. This involved the isolation and purification of A2U. A new technique was developed in the principal investigator's laboratory for removing contaminant urinary proteins from the final protein preparation. The purified A2U preparation was then used to develop monospecific antibodies for the detection of urinary, plasma and tissue-bound A2U. A rocket immunoelectrophoresis technique was developed in the principal investigator's laboratory to quantify plasma and urinary A2U.

Phase II was designed to investigate the mechanism of the nephrotoxic event from two perspectives: (1) to compare the association of A2U with the nephrotoxicity induced by pure hydrocarbon compounds and complex petroleum-based fuels; (2) to correlate alterations of the A2U with changes in the renal pathology. A decalin model was developed in collaboration with toxicologists at AAMRL/THT which could induce a reproducible nephrotoxicity in male, Fischer 344 rats. Histochemical, chromatographic and immunoelectrophoretic techniques are ongoing which should result in the most comprehensive evaluation of hydrocarbon-induced nephrotoxicity currently available.

This report is a final report for the principal investigators efforts at Hahnemann University, Philadelphia, PA (AFOSR 84-0283). However, the phase II goals are being pursued at the University of Illinois through the support of grant # AFOSR 86-0313.

MATERIALS AND METHODS

Laboratory Animals

A breeding colony of Fischer 344 rats was established by the principal investigator to provide a source of normal rat urine. The animals were housed in an AAALAC-approved facility and fed a standard rodent diet (Purina) by free choice.

Female, New Zealand white rabbits (3-5 kg) were used for the production of all immunologic reagents developed in this study. The animals were housed in an AAALAC-approved facility and fed a standard lagomorph diet (Purina) by free choice.

Isolation and Purification of A2U

Overnight urine specimens from young adult male Fischer 344 rats (100-200 days of age) were collected in a metabolic cage (Fisher Scientific). The urine was centrifuged and filtered through a 0.45 micrometer filter membrane in preparation for diafiltration. One volume of urine was diafiltered with ten volumes of 0.01 molar sodium phosphate buffer, pH 6.8 (PB) over a 5,000 dalton exclusion membrane (Amicon). This procedure replaced urinary salts with sodium phosphate salts and removed any urinary proteins with a molecular weight less than 5,000 daltons. Urine thus equilibrated with PB was added to a QMA anion exchange column (Waters) and the A2U peak zone recovered using gradient elution (0.01-0.50 molar PB). The A2U peak zone was reequilibrated with PB by diafiltration over a 5,000 dalton exclusion membrane in preparation for hydroxylapatite chromatography. The reequilibrated A2U peak zone was added to a hydroxylapatite matrix (Bio-Rad) and the purified A2U was recovered by gradient elution (0.01-0.3 molar PB).

Protein Concentration Determinations

All protein concentration determinations were performed using the Coomassie Blue dye-binding technique (Bradford, 1976) with bovine gamma globulin (Sigma) serving as the standard.

Production of Immunologic Reagents

Antisera to the various products of the A2U isolation and purification process were developed by an intramuscular adjuvant immunization procedure (Garvey, 1979). A Freund's complete adjuvant emulsion was made to 3 different antigens derived from young, adult male rat urine: (antigen #1) whole urine, (antigen #2) urine processed by anion-exchange chromatography, and (antigen #3) urine processed by anion-exchange and hydroxylapatite chromatography. One ml of the emulsion was divided into 5 different intramuscular injections given in the nuchal region and the thighs of each New Zealand White rabbit. The animals were rested for 4 weeks and a second set of intramuscular injections were given, with the antigen emulsion produced using Freund's incomplete adjuvant. The animals were rested for an additional 4 weeks and then bled to recover the resulting antiserum. The gamma globulin portion of the antiserum was purified by ammonium sulphate precipitation (Garvey, 1979). Residual ammonium sulphate salt was removed by dialysis and the final protein concentration of the purified antiserum made to 10 mg/ml.

Determination of A2U Purity

The purity of A2U isolated by the above technique was evaluated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and two dimensional immunoelectrophoresis (2-DIEP) (Hames, 1983).

The regression equation developed from the standard proteins was as follows: Y= 711 - 145x (correlation coefficient =.99). Column b represents A2U purified by combined anion-exchange and hydroxylapatite chromatography. The A2U band migrated 94 mm from the gel origin and thus had a calculated relative molecular weight of 18,300 daltons. Column d represents A2U purified by anion-exchange chromatography alone. In addition to the 18,300 dalton band, there are at least 2 contaminant protein bands in the 66,000 to 45,000 dalton range.

Isoelectric Focusing of A2U

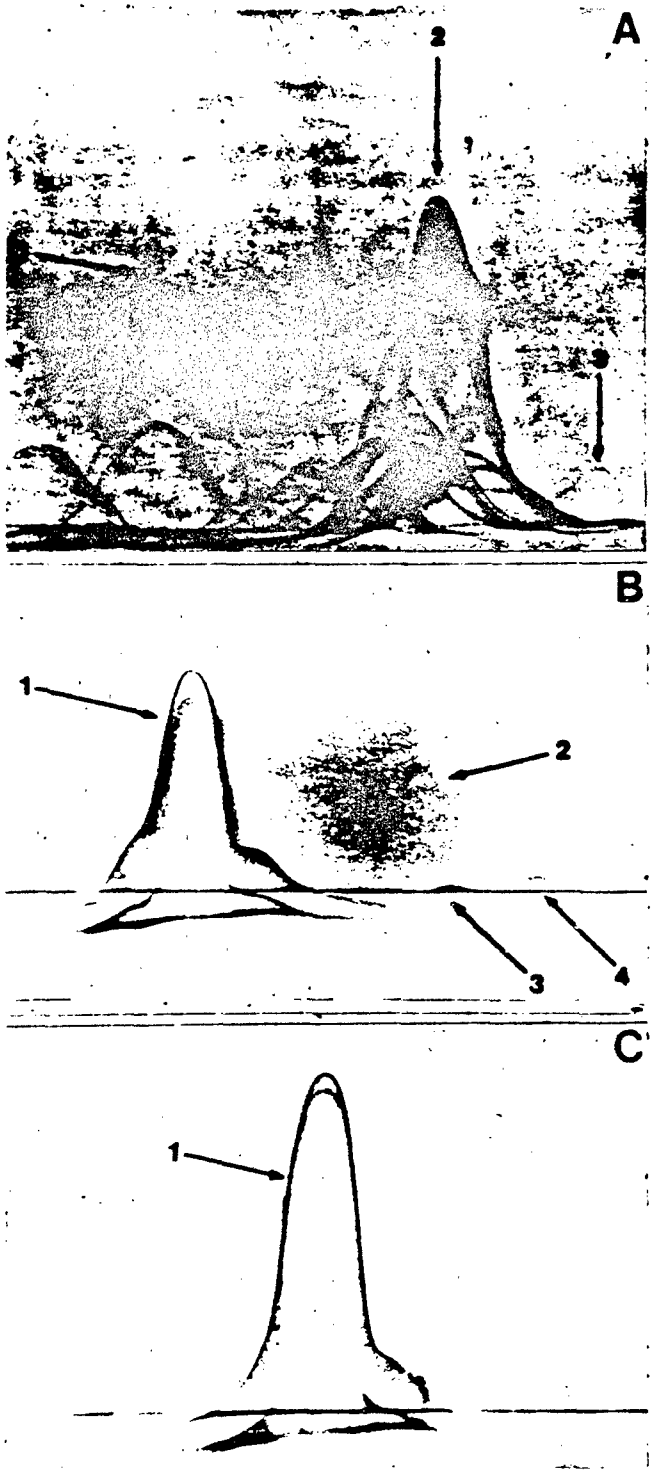
A2U purified by anion-exchange or by combined anion-exchange and hydroxylapatite chromatography was analyzed by isoelectric focusing (Figure 3). The electrophoresis was calibrated using standard proteins (FMC) with established isoelectric points (pI) ranging from pH 7 to pH 4. A2U was found to be a microheterogenous protein consisting of 5 isoelectric variants (pI = 5.1(#5), 5.3(#4), 5.4(#3), 5.5(#2), and 6.0(#1)). The A2U isoelectric variants with pI= 5.4 and 5.5 appear to occur in the highest concentration. Columns D and E of figure 3 can be used to compare the purity of the A2U recovered by anion-exchange alone or by combined anion-exchange and hydroxylapatite chromatography. Anion-exchange chromatography alone (column D) has at least two contaminating proteins (#6,pI=4.3; #7,pI=3.9) which are absent with combined anion-exchange and hydroxylapatite chromatography (column E).

Rocket Immunoelectrophoresis

Specific quantification of A2U in the complex mixture of urinary proteins was accomplished by rocket immunoelectrophoresis (Figure 4). Using this technique A2U could first be detected in the urine of 32 day old male rats. There appeared to be a gradual increase in the A2U concentration until the rat is approximately 300 days old, at which time the A2U concentration appears to decrease with age. Older rats (greater than 400 days old) appear to maintain an A2U concentration less than 1.0 mg/ml.

Decalin Exposure

Histopathologic evaluation of the animals exposed in experiment #1 (Page 10) revealed little difference between the experimental and control kidney specimens. Further studies at AAMRL/THT suggested a more prolonged exposure might be necessary (Olson, personal communication). There were consistent differences between the control and experimental groups in experiment #2 (Page 11) and experiment #3 (Page 12), indicating that the time course of decalin administration was important to the nephrotoxic process. In experiment #3 two of the animals in the high dose treatment group (animals # 04 and 05) developed clinical signs of marked weight loss and diarrhea.



urine sample = whole urine

antiserum = Rabbit anti whole urine

1=alpha 2U globulin

2=albumin

3=pre-albumin

urine sample = whole urine

antiserum = Rabbit anti anion exchange urinary proteins

1=alpha 2U globulin

2=contaminant alpha globulin

3=albumin

4=pre-albumin

urine sample = whole urine

antiserum = Rabbit anti anion exchange + hydroxylapatite urinary proteins

1= alpha 2U globulin

Figure 1. Two dimensional immunoelectrophoresis of rat urine preparations. 1st dimension anode towards the right, 2nd dimension anode towards the top.

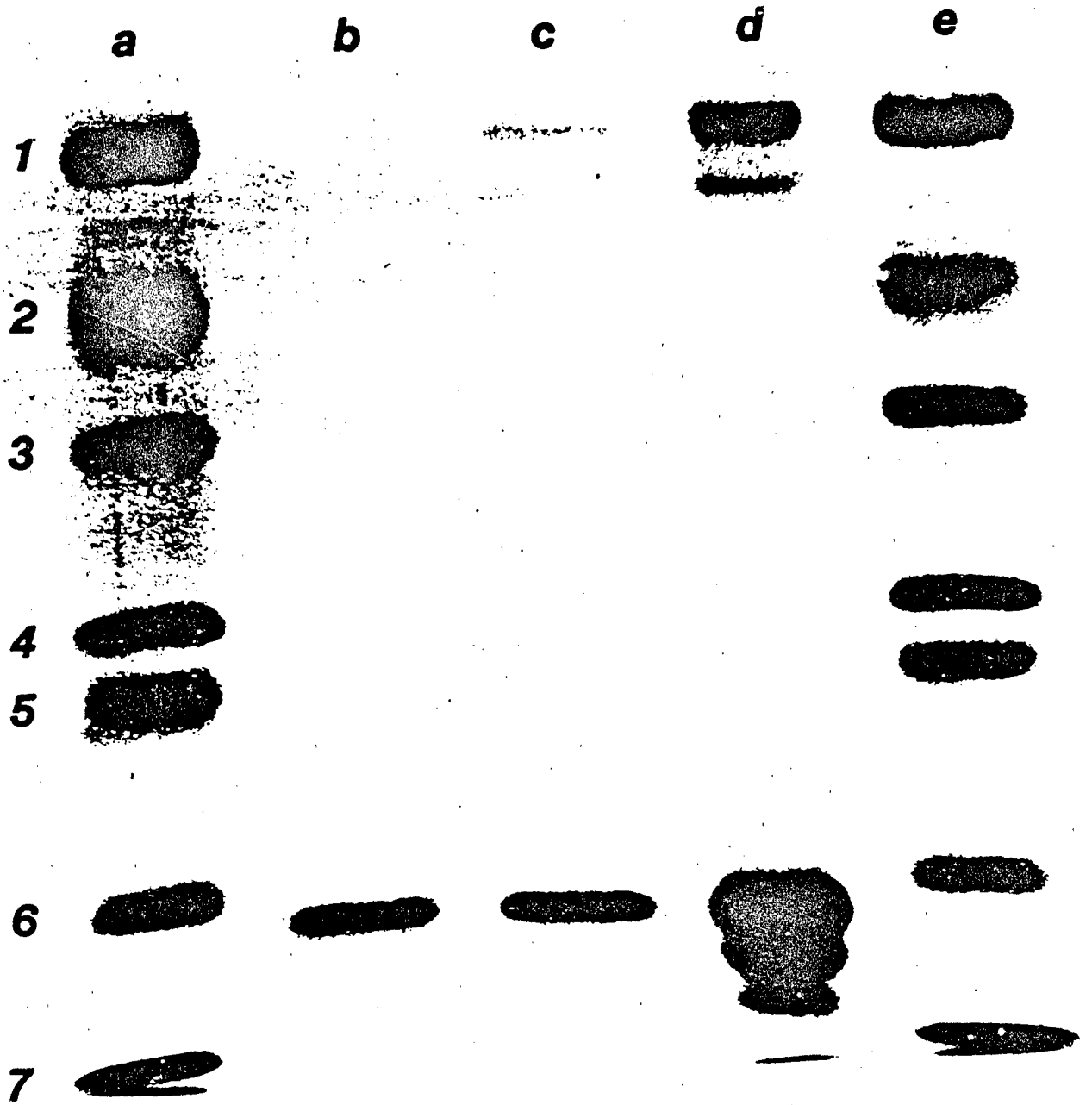


Figure 2. Sodium dodecyl sulphate-polyacrylamide gel of rat urinary proteins. Lanes a and e are standard proteins with the following molecular weight values (daltons); (1) 66,000, (2) 45,000, (3) 36,000, (4) 29,000, (5) 24,000, (6) 20,100, (7) 14,200. Lane b=A2U purified by anion exchange and hydroxylapatite chromatography. Lane c=A2U purified by anion exchange chromatography alone. Lane d=normal Fischer 344 male rat urine.

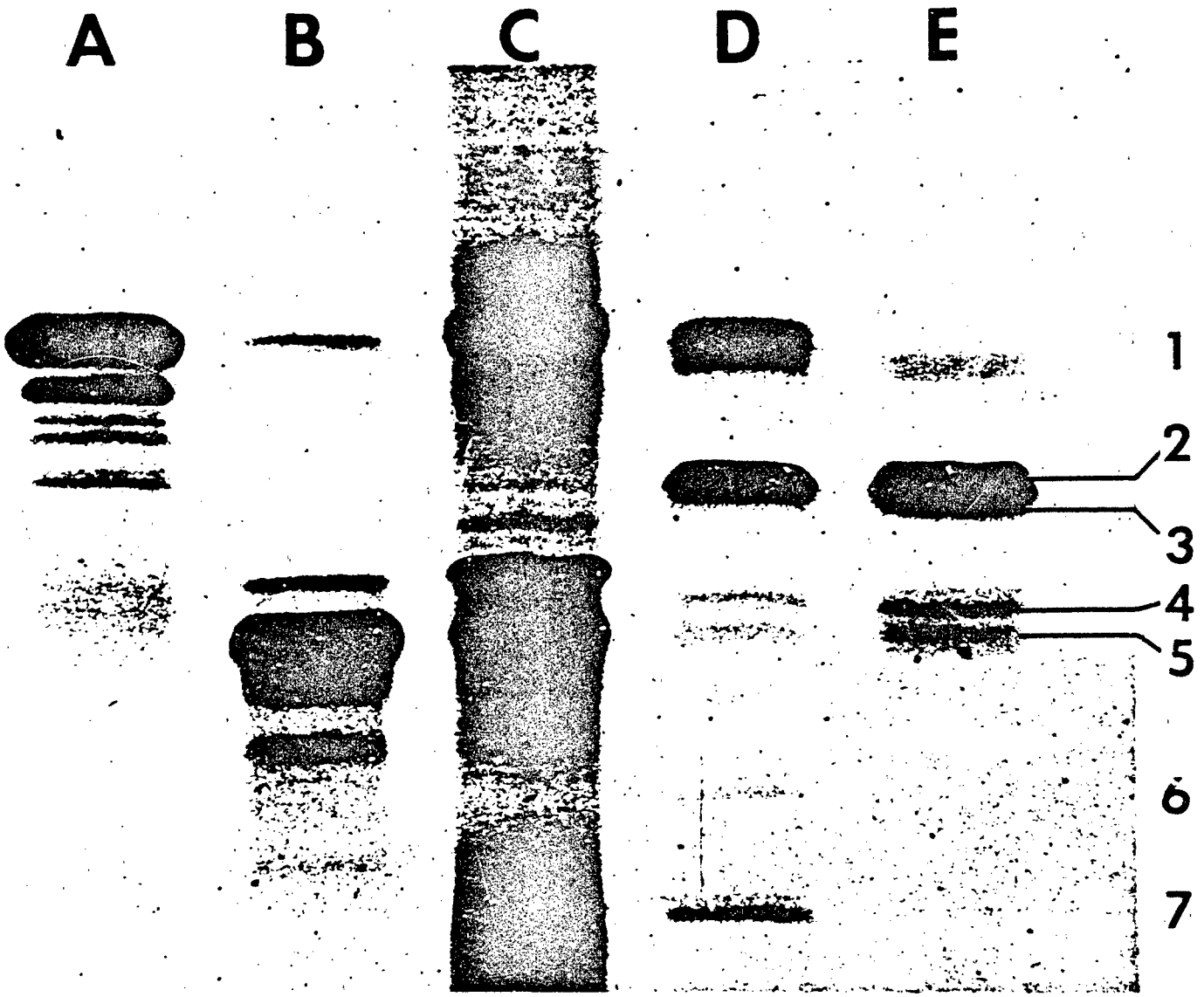


Figure 3. Isoelectric focusing pattern of A2U. Standard proteins used to calibrate the gel are shown in columns A, B, and C. The isoelectric variants of A2U purified by combined anion-exchange and hydroxylapatite chromatography are shown in column E and have the following pI values: (1)=6.0,(2)=5.5, (3)=5.4, (4)=5.3, and (5)=5.1. A2U isolated by anion-exchange chromatography alone (column D) contains the five variants listed above plus additional contaminant proteins #6 and #7.

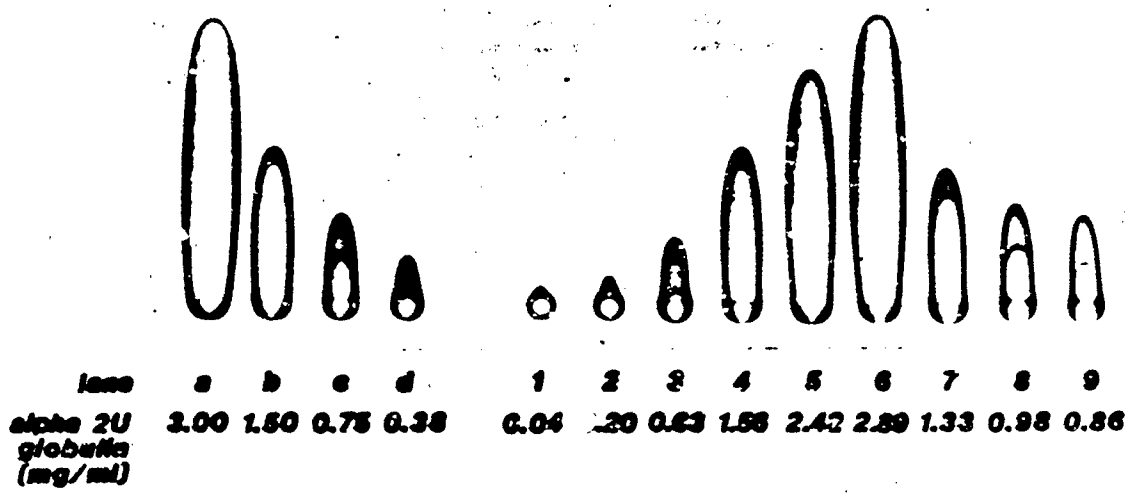


Figure 4. Quantification of A2U in Fischer 344 male rat urine by rocket immunoelectrophoresis. Lanes a through d are two-fold dilutions of a standardized A2U solution ranging from 3 mg/ml to 0.38 mg/ml. Lane 1=38 day old rat (0.04 mg/ml), lane 2=60 day old rat (0.20 mg/ml), lane 3=79 day old rat (0.63 mg/ml), lane 4=100 day old rat (1.56 mg/ml), lane 5=182 day old rat (2.42 mg/ml), lane 6=245 day old rat (2.89 mg/ml), lane 7=388 day old rat (1.33 mg/ml), lane 8=426 day old rat (0.98 mg/ml), lane 9=480 day old rat (0.86 mg/ml).

84-1 EXPERIMENT PATHOLOGY REPORT SUMMARY

TREATMENT	HYALIN DROPLETS IN PROX. EPI.	CORTICAL TUBULAR DILATION	PROXIMAL TUBULAR EPI. NECROSIS	CASTS	OTHER
H2O (2.0 ML/KG)					
84-1 (00)	2	1	+/-	-	-
84-2 (01)	1	+/-	-	-	-
84-3 (02)	2	1	-	OCC. (H)	-
84-8 (07)	1	1	-	-	-
DECALIN (1.0 ML/KG)					
84-4 (03)	2	1	1	OCC. (H/C)	
84-5 (04)	2	1	1	OCC. (H/C)	-
84-7 (06)	2	1	1-2	OCC. (H/C)	#1
84-9 (08)	2	1	2	OCC. (H/C)	#2
DECALIN (2.0 ML/KG)					
84-6 (05)	2	1	1-2	OCC. (H/C)	#2
84-10 (10)	1	+/-	-	-	-
84-11 (11)	1-2	+/-	1	-	-
84-12 (13)	2	1	1-2	OCC. (H/C)	#2

NOTE: OCC.=OCCASIONAL; (H)=HYALIN; (H/C)=HYALIN/CELLULAR
#1=FOCAL HYPERPLASIA OF UROTHELIUM
#2=PROXIMAL TUBULAR EPI.REGENERATION

SCORING- (+/-)=MINIMAL; (1)=MILD; (2)=MODERATE

85-1 EXPERIMENT PATHOLOGY REPORT SUMMARY

TREATMENT	HYALIN DROPLETS IN PROX. EPI.	CORTICAL TUBULAR DILATION	PROXIMAL TUBULAR EPI. NECROSIS	CASTS	OTHER
NO TX					
85-12 (36)	+/-	1	1	-	-
85-6 (42)	1	1	-	-	-
H20 (2.0 ML/KG)					
85-9 (39)	1	+/-	-	-	-
85-13 (43)	+/-	1	-	-	-
85-5 (49)	+/-	1	-	-	#2
85-14 (50)	+/-	1	-	-	#2
DECALIN (1.0 ML/KG)					
85-1 (26)	2	1	+/-	OCC. (H/C)	#1 #2,#4
85-7 (51)	2	1	1	OCC.(H/C)	#2
85-4 (40)	2	1	1	OCC. (H/C)	#2,#3
85-3 (41)	2	+/-	1	-	#2,#1
DECALIN (2.0 ML/KG)					
85-10 (24)	2	1	2	OCC. (H/C)	#2,#4
85-8 (48)	2	1	1	OCC.(H)	#2
85-2 (44)	2	1	1	OCC.(H)	#2,#3
85-11 (45)	2	1	1	OCC.(H/C)	#2

NOTE: OCC.=OCCASIONAL; (H)=HYALIN; (H/C)=HYALIN/CELLULAR
#1=FOCAL HYPERPLASIA OF UROTHELIUM; #2=PROXIMAL TUBULAR EPI. REGENERATION; #3=FOCAL PERIVASCULAR LYMPHOID AGGREGATES; #4=CORTICAL TUBULAR NEPHROLITHS (BASOPHILIC OVOID BODIES)

SCORING- (1)=MILD; (2)=MODERATE; (+/-)=MINIMAL

86.1 EXPERIMENTAL PATHOLOGY REPORT SUMMARY

TREATMENT	HYALIN DROPLETS IN PROX. EPI.	CORTICAL TUBULAR DILATION	PROXIMAL TUBULAR EPI. NECROSIS	CASTS	OTHER
H20 (2.0 ML/KG)					
86.4 (08)	+/-	+/-	-	-	-
86-12 (11)	+/-	1	-	-	-
86-1 (10)	+/-	+/-	-	-	-
86-6 (09)	+/-	1	-	-	-
DECALIN (1.0 ML/KG)					
86-3 (03)	2	1	1	OCC. (H)	#2
86-7 (00)	2	1	+/-	OCC. (H/C)	#2,#4
86-9 (02)	2	1	1	1 (H/C)	#2,#4
86-2 (01)	2	1	1	OCC. (H/C)	#2,#3
DECALIN (2.0 ML/KG)					
86-10 (04)	2-3	1	1-2	2 (H/C)	#2,#3 #4
86-8 (05)	2	2	1-2	1 (H/C)	#2,#3 #4
86-11 (06)	2	1	2	1 (H/C)	#2
86-5 (07)	2	1	2	2 (H)	#2,#4

NOTE: OCC.=OCCASIONAL; (H)=HYALIN; (H/C)=HYALIN/CELLULAR; #1=FOCAL HYPERPLASIA OF UROTHELIUM; #2=PROXIMAL TUBULAR EPI. REGENERATION; #3=FOCAL PERIVASCULAR LYMPHOID AGGREGATES; #4=CORTICAL TUBULAR NEPHROLITHS (BASOPHILIC OVOID BODIES)

SCORING- (+/-)=MINIMAL; (1)=MILD; (2)=MODERATE; (3)=SEVERE

DISCUSSION

Young adult male rat urine is a complex mixture of at least 20 urinary proteins, of which A2U is the major single protein element (Figure 1A). A goal of this project was to develop antisera which would specifically detect A2U in urine without cross reacting with other urinary proteins. Cross reactive antibodies against contaminant urinary proteins would be present in an given antisera if the antigenic preparation used to induce that antisera contained urinary proteins other than A2U. Immunologic reagents containing antibodies against albumin would be particularly detrimental to accurate interpretation of test results as albumin is the second most prevalent protein in young adult male rat urine.

A useful application of 2-DIEP is to evaluate the specificity of an antisera against a particular antigen. The method most often cited in the literature for the isolation of A2U is based on anion-exchange chromatography, and results in a preparation contaminated with alpha globulins, albumin, and pre-albumins. Immunization with A2U recovered by such a method results in a non-specific antisera (Figure 1B). A technique combining diafiltration, anion-exchange and hydroxylapatite chromatography was developed in the principal investigator's laboratory to isolate A2U from urine specimens. Immunization with A2U isolated by this method results in an A2U preparation free of contaminating urinary proteins (Figure 1C).

The controversy regarding the association of A2U with hydrocarbon-induced nephrotoxicity may stem from the use of non-specific immunologic reagents which cross react with urinary proteins other than A2U. The specific antisera developed in this study is currently being used to address the A2U controversy from two perspectives: (1) to monitor plasma and urinary changes in A2U with experimental hydrocarbon exposure using a rocket immunoelectrophoretic method (Figure 4) and (2) to evaluate the association of A2U with the renal lesion using immunohistochemical techniques. This effort is being continued through the support of grant AFOSR-86-0313.

Fischer 344 rat A2U has been reported to occur in two distinct molecular forms which differ in their apparent molecular weight (A2U/a=18,800 daltons and A2U/b=18,000 daltons) (Chatterjee, 1982). The SDS-PAGE analysis of Fischer 344 rat A2U in the present study revealed a single band with an apparent molecular weight of 18,300 daltons (Figure 2). By using a more sensitive analysis technique such as high resolution 2-DIEP analysis the A2U molecule was shown to contain two major A2U subunits (Figure 1C). These two components differ in their 2-DIEP anodic migration in the second dimension (anode located towards the top of the gel). The two molecular forms of A2U can be further identified by a yet more sensitive method such as isoelectric focusing (Figure 3). Component 3 (pI=5.4) of Figure 3 represents the more anodic peak component in Figure 1C, whereas component 2 (pI=5.5) of Figure 3 represents the less anodic peak in Figure 1C. Although the referenced study and the present study both demonstrated that Fischer 344 rat A2U occurs in two distinct molecular forms, the present study detected only one band by SDS-PAGE. 2-DIEP is a more sensitive test of molecular heterogeneity (Axelsen, 1973) than SDS-PAGE therefore one source of the discrepancy in molecular weights might be the occurrence of contaminant urinary proteins in the SDS-PAGE experiment of the referenced study.

In addition to the two major isoelectric variants of A2U (pI=5.5 and 5.4), the present study demonstrated three minor isoelectric variants (pI=5.1, 5.3, and 6.0; Figure 4, #5, #4, and #1,respectively). Wistar male rat A2U has been reported to consist of five isoelectric variants (pI=7.8, 6.1, 4.9, 4.1, and 3.7) (Roy, 1983), with the 6.1 and the 4.9 variants being the major components. The discrepancy between the present study and the referenced study may reflect rat strain variation. A2U strain variation is currently being studied by the principal investigator using Sprague-Dawley and Long-Evans rats.

There are no studies in the literature regarding the isoelectric variant profile of serum or urinary A2U and the expression of hydrocarbon-induced nephrotoxicity. However, the isoelectric variant profiles of certain human urinary proteins have provided new insight into several nephropathologic processes. Alpha-1-microglobulin (another low molecular weight urinary protein) from normal human urine has two major isoelectric variant subunits (pI=4.2 and 4.8) which occur in a 1:2 ratio. In certain types of renal tubular proteinuria, the isoelectric variant profile of alpha-1-microglobulin changes (pI= 4.1 and 4.6) as does the subunit ratio (1:5) (Bernier, 1980). A similar situation occurs in multiple myeloma, where the severity of the renal tubular lesion appears to be related to the isoelectric point (pI) of the immunoglobulin light chain (Bence Jones Protein) produced in this disease (Coward, 1984).

This project has been successful in reaching its goals and has developed several elements which are currently being used by the principal investigator to study the association of A2U with hydrocarbon-induced nephrotoxicity:

(1) A pure A2U preparation without the contaminant urinary proteins seen with standard recovery procedures. This preparation will allow the first accurate evaluation of Fischer 344 rat A2U isoelectric variant profile changes associated with a hydrocarbon-induced renal lesion.

(2) A specific antisera probe for detecting A2U in the complex protein mixture of rat urine. The rocket immunoelectrophoresis technique for A2U developed in this study is the first reported quantitative assay specific for A2U.

(3) A reproducible procedure for inducing the renal lesion with a pure hydrocarbon compound (Decalin).

The scientific advances attained through the support of grant # AFOSR-84-0283 are currently being continued and expanded through the support of grant #AFOSR-86-0313, and should provide new insights into the controversial aspects of hydrocarbon-induced nephrotoxicity.

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ABSTRACTS AND PUBLICATIONS

1. Eurell, T.E., Olson, C.T., and Hobson, D.W. A new technique for the isolation and purification of urinary alpha-2U globulin from Fischer 344 rats. The Toxicologist (6)#1:204, 1986.

2. Hobson, D.W., Olson, C.T., Eurell, T.E., Bruner, R., and Uddin, D. Effect of castration on development of 2,3,4 trimethylpentane induced hydrocarbon nephropathy in male F344 rats. The Toxicologist (6)#1:173, 1986

3. Eurell, T.E., and Olson, C.T. A new technique for the isolation and purification of urinary alpha-2U globulin from Fischer 344 rats. (In preparation).

4. Eurell, T.E., Henningsen, G., and Olson, C.T. Comparison of strain differences between male rat urinary alpha-2U globulin. (In preparation).

PROFESSIONAL PERSONNEL ASSOCIATED WITH THE RESEARCH EFFORT

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3. G. Henningsen, D.V.M., Ph.D.-Toxicologist and Chief, AAMRL/THT Wright Patterson AFB, OR.

INTERACTIONS

Consultation with AAMRL/THT toxicologists and pathologists at Wright-Patterson AFB:

(1) January 23-25, 1985
(2) April 16-18, 1986